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Complex origins indicate a potential bridgehead introduction of an emerging amphibian invader (*Eleutherodactylus planirostris*) in China

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Abstract

Identifying the origins of established alien species is important to prevent new introductions in the future. The greenhouse frog (*Eleutherodactylus planirostris*), native to Cuba, the Bahamas, and the Cayman Islands, has been widely introduced to the Caribbean, North and Central America, Oceania and Asia. This invasive alien amphibian was recently reported in Shenzhen, China, but the potential introduction sources remain poorly understood. Based on phylogenetic analysis using mitochondrial 16S, COI and CYTB sequences, we detected a complex introduction origin of this species, which may be from Hong Kong, China, the Philippines, Panama and Florida, USA, all pointing to a bridgehead introduction. In

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addition, the nursery trade between the four countries or regions and mainland China from 2011 to 2020 was also significantly higher than other areas with less likelihood of introductions, which supported the molecular results. Our study provides the first genetic evidence of the potential sources of this emerging amphibian invader in mainland China, which may help develop alien species control strategies in the face of growing trade through globalization.

Keywords
Amphibian, biosecurity, bridgehead introduction, invasive species, phylogenetics

Introduction

Alien species invasion has been a major threat to global biodiversity, the economy and human health in the current era of Anthropocene (Pyšek et al. 2020). The rate of alien species invasion has substantially accelerated over the past century and is particularly concerning, because human-mediated introductions have reached unprecedented intensity (Seebens et al. 2017), which is projected to continue to increase in the next 50 years under sustained globalization (Seebens et al. 2021). Along the "introduction-establishment-spread" continuum of biological invasions (Blackburn et al. 2011), managing early risks at the introduction stage is considered one of the most effective mitigation strategies (Fournier et al. 2019). With the rapid development of molecular tools, identifying the origins of alien species based on molecular markers is critically important to understand the potential introduction process and thus prevent new potential invasions in the future (Estoup and Guillemaud 2010; Hudson et al. 2021), which has been widely used to develop control strategies for invasive alien species (Bai et al. 2012; Liebl et al. 2015; Wang et al. 2017).

Alien amphibians have been a particular conservation and environmental concern due to their substantial impacts on native species through predation, competition, and the spread of notorious wildlife diseases such as the chytridiomycosis panzootic (Liu et al. 2013; Kraus 2015). Additionally, there is general rapid anthropogenic introduction and dispersal of alien amphibians due to their close associations with human activities through the pet trade and aquaculture (Kraus 2009; Liu et al. 2014; Capinha et al. 2017; Capinha et al. 2020). The greenhouse frog (*Eleutherodactylus planirostris*), native to a few islands of the Caribbean, Cuba, and the Cayman Islands, the Bahamas, and the British Overseas Territory, is one such species that has been widely introduced in more than 10 states of the USA (Kraus et al. 1999; Somma 2022), Nicaragua (Heinicke et al. 2011), Mexico (Cedeño-Vázquez et al. 2014), Panama (Crawford et al. 2011), Jamaica (Pough et al. 1977), and Guam (Christy et al. 2007). In China, this species was first recorded in Hong Kong in 2000 (Lee et al. 2016), and then was found in Shenzhen in 2017 (Lin et al. 2017). This species is regarded as one of the most successful amphibian invaders with high population densities (e.g., 12,500 frogs/hectare in Hawaii (Olson and Beard 2012)) and diverse prey across a wide range of

invertebrates (e.g., 129,000 individuals/hectare/night (Olson and Beard 2012)), posing a high predation threat to insect populations and competition for food with other sympatric frogs in invaded areas (Kraus et al. 1999; Olson and Beard 2012). Notably, it is a direct-developing frog without a free-swimming larval stage and thus is highly adaptable to various terrestrial and aquatic habitats (AmphibiaWeb 2022). In addition, owing to its tiny body size (16–32 mm SVL), it is extremely well-suited to introduction to new ranges as a stowaway through imported plants and landscaping materials (Kraus and Campbell 2002). This unobserved and unintentional introduction makes it difficult to identify the source populations, which is nevertheless crucial for early prevention strategies to stop future introductions and may be possible with the aid of molecular tools (Ficetola et al. 2008).

Heinicke et al. (2011) first revealed the origin of invasive *E. planirostris* found in Florida might lie in western Cuba using a phylogenetic analysis method based on three gene markers (CYTB, RAG1 and PMOC). Later, studies of several invasive populations, such as those in Panama (2 samples, Andrew and Alonso 2011) and Hong Kong, China (2 samples, Lee et al. 2016), uncovered a bridgehead introduction that originated from the already established Florida populations, based on partial fragments of the 16S rRNA gene. Bridgehead introduction usually tends to cause loss of genetic diversity (Bertelsmeier and Keller 2018), which was evidenced by the observed extremely low genetic diversification of mitochondrial genes (CYTB, 16S rRNA gene or COI) from the populations in Mexico, the Philippines, Hawaii and Florida, USA (Cedeño-Vázquez et al. 2014; Contreras-Calvario et al. 2018; Que et al. 2020). These studies have provided striking examples of the possible introduction processes of different invasive populations of the greenhouse frog around the world. However, the potential origins of its emerging population in Shenzhen, China are still unclear. Shenzhen is located in the Guangdong-Hong Kong-Macao Greater Bay Area, which is an important region with a highly developed horticulture, gardening, and landscaping industry that provides an ideal opportunity for the introduction of greenhouse frogs through horticultural transportation. We speculated that Hong Kong, China, may be the most likely source of the Shenzhen population due to its close geographical location, similar climate, and frequent trade. However, the possibility of introduction from other countries and regions cannot be ruled out due to accelerating international trade, transportation, and infrastructure construction (Ding et al. 2008; Huang et al. 2012; Liu et al. 2019).

To fill this knowledge gap, we used phylogenetic analyses to explore the possible introduction routes of the Shenzhen population based on data from existing native and invasive populations and all available molecular sequences across the world. We aimed to provide timely insights into the source of this emerging frog invader in mainland China and contribute to the development of a sustainable Chinese biosecurity strategy against biological invasions, especially in regions such as the Guangdong-Hong Kong-Macao Greater Bay Area with growing social and economic activities for the prevention of biological invasions.

Methods

Field Sampling

Field surveys were conducted in Shenzhen, China on September 2–13, 2021. The third toe of the right hind foot from each postmetamorphosis greenhouse frog was clipped, and the tissue samples were preserved separately in 95% ethanol and stored at –20 °C in the laboratory (Suppl. material 1: Table S1). A total of four samples were used for the phylogenetic analysis.

We obtained all published sequences of the greenhouse frog, including a total of 71 CYTB sequences, 13 COI sequences and 18 16S sequences from NCBI; All newly obtained sequences were deposited in GenBank (CYTB: OP554912-OP554915; COI: OP548504-OP548506, OP548508; 16S: OP547501, OP547876-OP547878) (Suppl. material 1: Table S1), along with one outgroup sequence from *Osteopilus septentrionalis* from Heinicke et al. (2011).

DNA extraction and amplification

Genomic DNA was extracted using the Universal Genomic DNA Kit (catalog no. CW2298M; Beijing, CoWinBiotech Co., Ltd, Beijing, China) following the manufacturer's instructions. To infer the potential geographic origin of the sampled individuals, we amplified one diagnostic mitochondrial marker corresponding to a portion of the protein encoding the 16S region, a portion of the protein encoding COI (Simon et al. 1994; Che et al. 2012), and a portion of the protein encoding the CYTB region, which were designed for this study using Primer 5 software (Lalitha 2000). The amplification conditions are shown in Suppl. material 2: Table S2. The PCR products were then separated by electrophoresis on 2% agarose gels. The resulting PCR products were directly sequenced by Beijing Liuhe Bgi Co., Ltd, Beijing, China, using the same primers for amplification. All sequences were tested for quality and calibrated manually using SeqMan in the LASERGENE 7.0 software package (Ahmed et al. 2016) to generate consistent sequences. All three genes obtained for each specimen sequence were compared with the available homologous sequences from GenBank (https://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the default parameters. All the obtained consensus sequences were aligned using the default parameters in Clustal X 1.81 (Thompson et al. 1997), and then MEGA 7.0 was used to compare the sequences of each gene (Tamura et al. 2011).

Data analysis

Phylogenetic relationships of the greenhouse frog were reconstructed based on 16S sequence, COI sequence and CYTB sequence data using maximum likelihood (ML) and Bayesian (BI) analyses, respectively. The 'best' model of sequence evolution for the sequences was inferred using the Akaike Information Criterion (AIC) as implemented

in jModelTest 2 (Darriba et al. 2012). The best model was GIR+G based on 16S data, Since COI gene and CYTB gene were coding genes, based on this selection, we used DAMBE software (Xia and Lemey 2009) to screen each codon of COI and CYTB sequences, and used ModelTest software to calculate the optimal evolutionary model of each codon: TrNef for the first codon position of COI; F81 for the second codon position of COI; HKY+I for the third codon position of COI; TrH for the first codon position of CYTB; JC for the second and third codon position of CYTB. We ran an ML search using RAxML version 8.2.4 and assessed the results using nonparametric bootstrap resampling with 1000 replicates (Stamatakis 2014). Bayesian phylogenetic analyses were conducted using MrBayes 3.2 (Ronquist et al. 2012). According to the selected nucleotide replacement model, we implemented two independent runs at the default calorific value and used four MCMC chains each time, including three hot chains and one cold chain. All the data analyses were run for 1000 million generations and were sampled every 200 generations. Bayesian posterior probability values were estimated from the sampled trees that remained after the first 25% of trees were discarded as burn-in. Convergence was assured by an average standard deviation of split frequencies below 0.01 and accurate parameter estimates as indicated by estimated sample sizes above 200 and potential scale reduction factor values close to 1 in Tracer v1.5 (Rambaut and Drummond 2007). The haplotypes of each gene were calculated in DnaSP 5.10 (Librado and Rozas 2009) (Suppl. material 1: Table S1). Haplotype NETWORK graphs were generated using POPART 1.7 software (Ropiquet et al. 2015).

Validation of molecular analyses using nursery trade data

Considering the fact that the greenhouse frog was mainly transported through nursery trade (Kraus 2009; Lee et al. 2016; Lin et al. 2017), in order to validate the results based on molecular data, we further collected the available nursery trade volumes imported from all the candidate countries or regions to mainland China from different databases such as the United Nations Commodity Trade Statistics database (https://comtrade.un.org/, Suppl. material 3: Table S3). Since the greenhouse frog was first discovered in Shenzhen, China, in 2017 (Lin et al. 2017), we collected a total of 10-year bilateral trade data from 2011 to 2020. We then used a Kruskal-Wallis test to compare the 10-year trade volume between mainland China and the countries or regions with the most likely sources including Hong Kong, China, the Philippines, Panama, and Florida, USA and the other areas with less introduction likelihood based on molecular data.

Results

Our 16S sequence data set consisted of 550 bp from all 4 individuals in a matrix of 137 variable sites. Combined with the results of the haplotype network, ML and BI trees constructed from the sequences of three genes indicated that the Shenzhen population may be from Hong Kong, China, the Philippines, Panama and Florida, USA.

For instance, the samples from Shenzhen were found to share the same haplotype with populations in Hong Kong, China, the Philippines, Panama, and Florida, USA, based on the sequences of the 16S gene (Fig. 1). Similarly, the COI sequence data set consisted of 596 bp from all 4 individuals in a matrix of 149 mutation locus, which also supported the conclusion that the greenhouse frog in Shenzhen may originate from the populations in the Philippines and Panama, which share the same haplotype (Fig. 2). The CYTB sequence data set consisted of 635 bp from all 4 individuals in a matrix of 174 mutation locus. However, the CYTB sequences showed that the greenhouse frog from Shenzhen had a distinct haplotype, and it was closest to the haplotype shared by Florida, Hawaii, USA, Matanzas in Cuba, and the Philippines, which suggested that the individuals of Shenzhen population may have originated from one or more of these areas (Fig. 3). The bilateral trade analysis further showed that the volumes of importing nursery trade in 2011–2020 from the molecular-based likely source areas including Hong Kong, China, the Philippines, Panama, and Florida, USA, was indeed significantly higher than the other areas with less likelihood of introductions (Bahamas; Cuba; Cayman Islands, UK; Turks and Caicos Islands, UK; Jamaica; Nicaragua; Hawaii, USA) (Kruskal-Wallis test, $P = 0.0001175$).

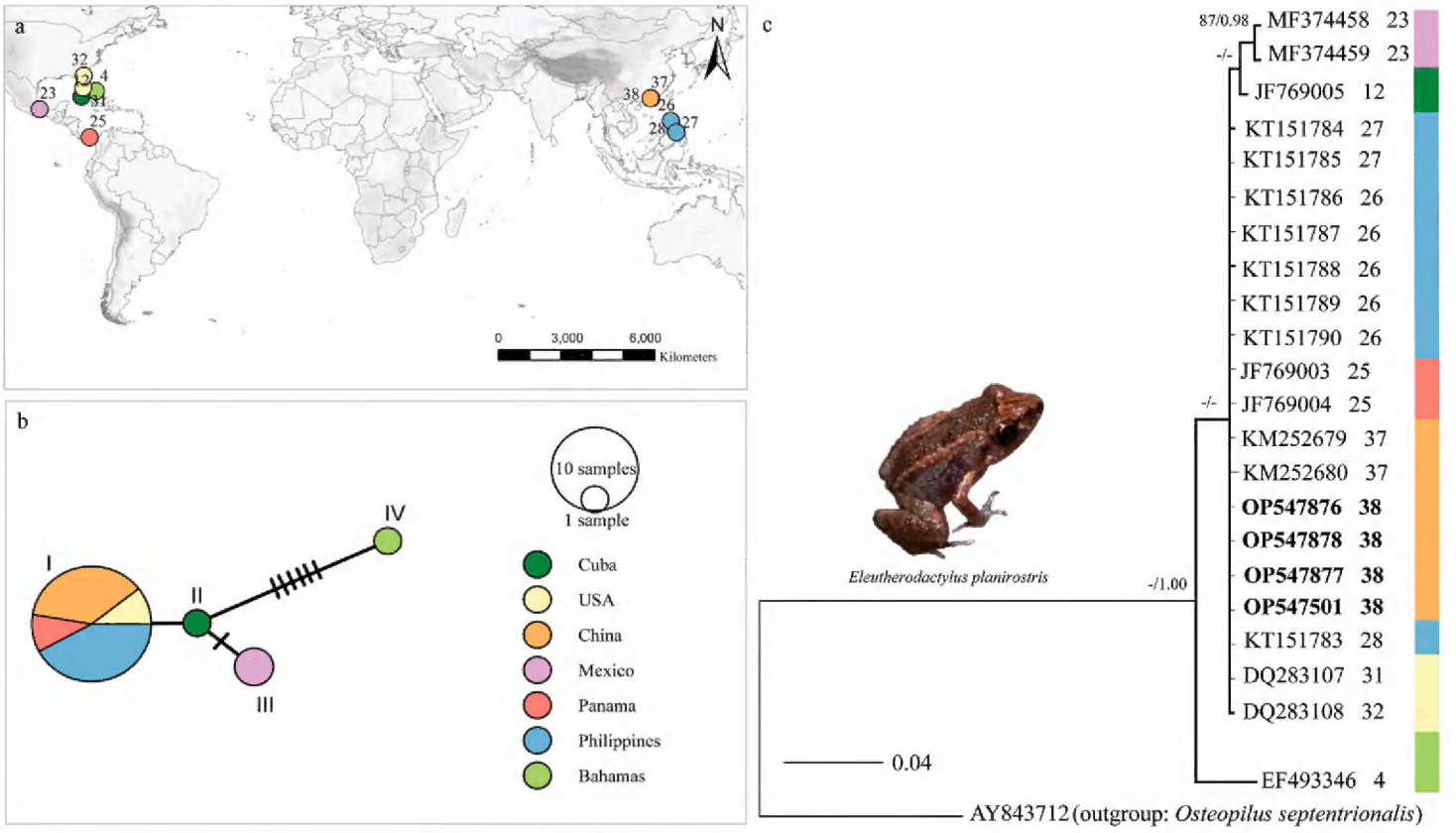

Figure 1. a sample distribution and results for the mitochondrial 16S sequences; each number represents a different sequence location **b** relationship among the network of haplotypes of the greenhouse frog (*Eleutherodactylus planirostris*). The size of the circles is proportional to haplotype frequency. Each color represents a locality/country **c** bayesian/maximum Likelihood phylogenetic tree of the greenhouse frog inferred from a fragment of the 16S gene. "-" denotes low support by Bayesian posterior probabilities (BPP < 95%) and bootstrap support (BS < 70%). Colors indicate mitochondrial lineages, and each color represents a different country: Cuba (dark green), Bahamas (aqua), USA (light yellow), Mexico (purple), Panama (red), the Philippines (blue), and China (orange). *E. planirostris* image: from http://www.amphibiachina.org/.

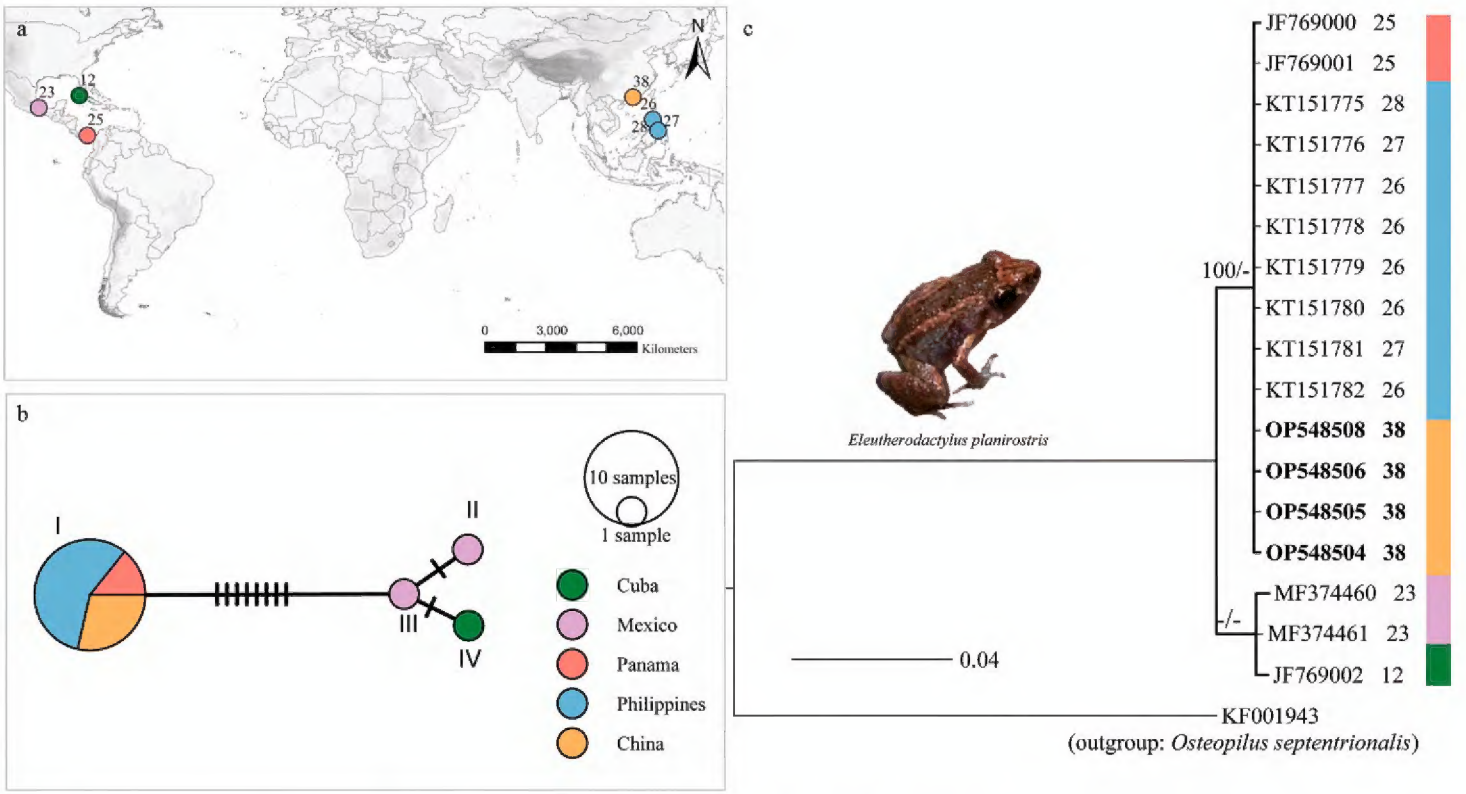

Figure 2. a sample distribution and results for the mitochondrial COI sequences; each number represents a different sequence location **b** relationship among the network of haplotypes of the greenhouse frog (*Eleutherodactylus planirostris*). The size of the circles is proportional to haplotype frequency. Each color represents a locality/country **c** bayesian/maximum likelihood phylogenetic tree of the greenhouse frog inferred from a fragment of the COI gene. "-" denotes low support by Bayesian posterior probabilities (BPP < 95%) and bootstrap support (BS < 70%). Colors indicate mitochondrial lineages, and each color represents a different country: Cuba (dark green), Mexico (purple), Panama (red), the Philippines (blue), and China (orange). *E. planirostris* image: from http://www.amphibiachina.org/.

Discussion

To the best of our knowledge, this is the first quantitative study on the potential introduction source of the emerging global amphibian invader (the greenhouse frog, *E. planirostris*) in mainland China. According to the standard of Heinicke et al. (2011), our results using 16S and COI identified two lineages of the greenhouse frog (i.e., an eastern lineage from eastern/central Cuba, the Bahamas and the Cayman Islands, and a western lineage from western Cuba and Florida, USA), and the samples we obtained from the Shenzhen population belong to the western lineage (Figs 1–3). In the western lineage, previous studies suggested that the greenhouse frog in Hong Kong, China and the Philippines originated in Florida (Lee et al. 2016; Que et al. 2020); our result thus indicates that the original source of the greenhouse frog in Shenzhen may be Florida, USA, which warrants further investigation using more samples collected at each site and based on more powerful genetic information, such as genomes generated by next-generation sequencing techniques (Blumenfeld et al. 2021). Mitochondrial DNA used in our present study may, in particular, have a limited ability to track recent invasions because this marker requires accumulation of variation over long timescales (Browett et al. 2020). The whole-genome or molecular marker with high mutation rate (such as microsatellites

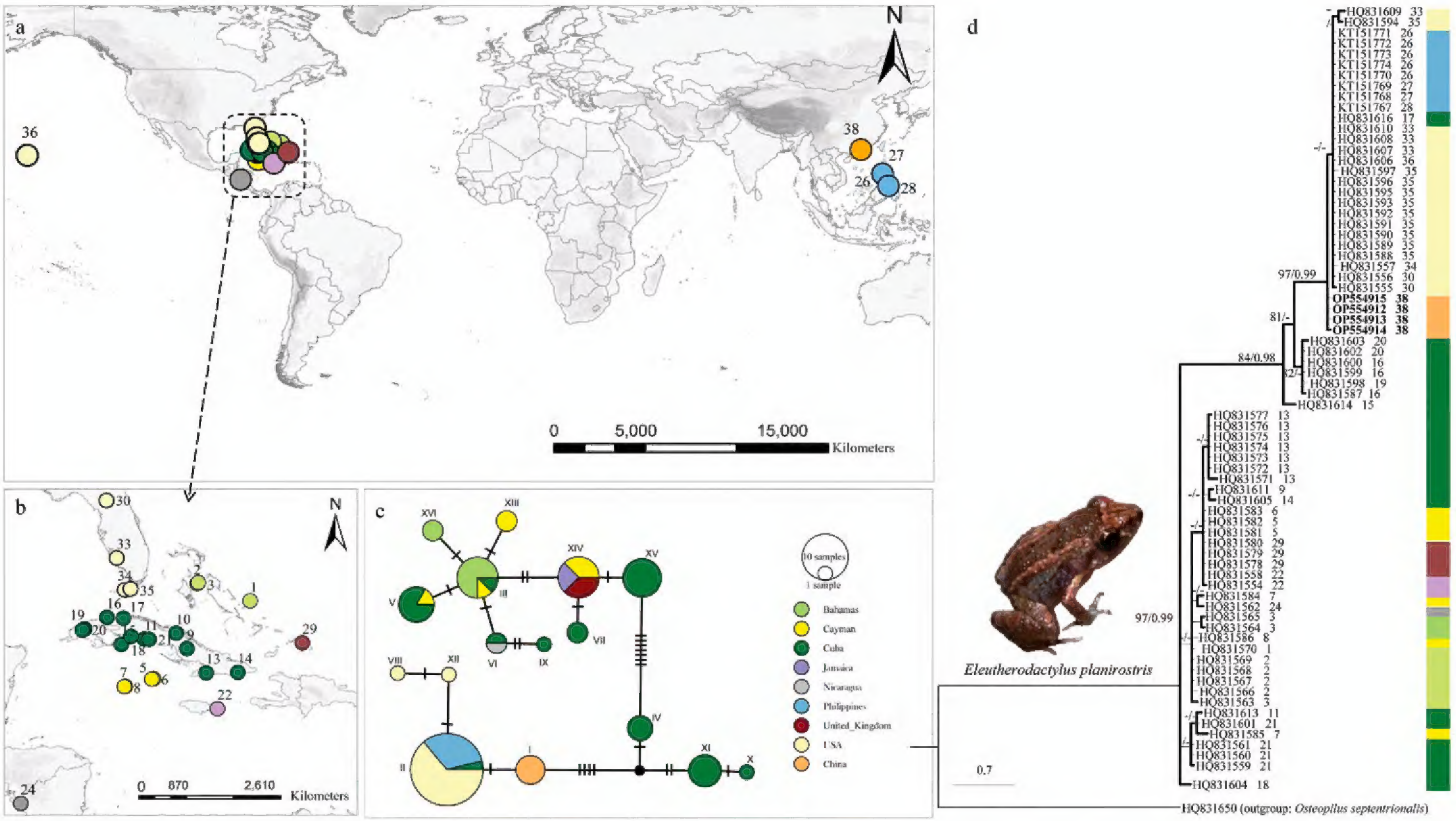

Figure 3. a, b sample distribution and results for the mitochondrial CYTB sequences. Each number represents a different sequence location **c** relationship among the network of haplotypes of the greenhouse frog (*Eleutherodactylus planirostris*). The size of the circles is proportional to haplotype frequency. Each color represents a locality/country **d** bayesian/maximum likelihood phylogenetic tree of the greenhouse frog inferred from a fragment of the CYTB gene. "-" denotes low support by Bayesian posterior probabilities (BPP < 95%) and bootstrap support (BS < 70%). Colors indicate mitochondrial lineages, and each color represents a different country: Cuba (dark green), Bahamas (aqua), Cayman (yellow), USA (light yellow), Jamaica (dark purple), UK (dark red), Nicaragua (gray), the Philippines (blue), and China (orange). *E. planirostris* image: from http://www.amphibiachina.org/.

and single nucleotide polymorphisms, SNPs) can help improve the analysis accuracy (Ellegren 2004; Li 2011; Rius et al. 2015). However, data on these higher resolution markers of the greenhouse frog are not available yet from Genbank. We suggest that further investigations with closer international collaborations can obtain cross-border sample collections to facilitate a more robust identification of introduction sources of this global frog invader. We therefore encourage future studies using microsatellites or SNP markers to support or refute our conclusions. Nevertheless, our analyses based on the volume of the bilateral nursery trade between different candidate countries or regions and mainland China supported our molecular analysis as the trade volumes of several higher likely source areas such as Florida, USA, Philippines, Hong Kong, China and Panama, is indeed higher than other areas with lower introduction likelihood.

Interestingly, all the candidate source populations identified in the present study are located in the nonnative ranges of the greenhouse frog, supporting the potential bridgehead introductions, which have been observed in several other invasive populations around the world (e.g., Andrew and Alonso 2011; Lee et al. 2016). The bridgehead effect can often reduce the genetic diversity of subsequent invasive populations after experiencing bottleneck events (Blumenfeld et al. 2021). However, bridgehead populations

have been found to effectively reduce inbreeding depression by removing those deleterious alleles (Facon et al. 2011) and increasing the rate of adaptive evolution of those traits, promoting their invasion of novel ranges (Bertelsmeier and Keller 2018). Therefore, the bridgehead effect of alien species has been observed to be increasingly common across taxa, especially for invasive insects (Correa et al. 2019; Javal et al. 2019; Sherpa et al. 2019). The increasing bridgehead introduction modes of alien species imply the importance of applying transport network theory by incorporating trade and animal translocations to obtain a full picture of the alien species introduction process (Banks et al. 2015).

In general, the global invasive populations of the greenhouse frog have always been reported in large cities or in localities with great commercial nursery trade volumes (Contreras-Calvario et al. 2018), which is one important pathway involved in the human-mediated movement of alien herpetofauna (Kraus 2009). There has been a long history of nursery trade in the Pearl River Delta region of Guangdong Province and Hong Kong, China. For example, in 2015, over 100,000 kg of plants or parts of plants were exported from Hong Kong, China, to tropical or subtropical countries/or regions, including Australia, mainland China, Macau, China, Malaysia, Singapore, Thailand, Taiwan, China and Vietnam (Census and Statistics Department, Hong Kong SAR 2015). Such a high volume of horticulture might, therefore, have posed a high risk of alien species invasions, especially the alien herpetofauna. We therefore suggest that more strict quarantine policies and early warning frameworks should be made to prevent the continued incursions of alien species.

Conclusion

We provided genetic evidence on the potential introduction sources of an emerging amphibian invader (*E. planirostris*) in China, which is further validated using international nursery trade of different alternative countries or regions with mainland China. We observed multiple introduction candidate sources, which all indicated a bridgehead introduction. Overall, these findings demonstrate the complexity of the greenhouse frog introductions from their already invaded ranges to China and stress the importance of developing stricter monitoring strategies to mitigate the stowaway introduction of this global amphibian invader from different areas worldwide.

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Supplementary material 1

Table S1

Authors: Yanhua Hong Yanhong He Zhiqiang Lin, Yuanbao Du, Shengnan Chen, Lixia Han, Qing Zhang, Shimin Gu, Weishan Tu, Shengwei Hu, Zhiyong Yuan, Xuan Liu

Data type: occurences, phylogenetic

Explanation note: Sequence location and distribution for genetic samples of *Eleutherodactylus planirostris* used in this study. We obtained all published sequences of the frog, including a total of 71 CYTB sequences, 13 COI sequences and 18 16S sequences from NCBI.

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Link: https://doi.org/10.3897/neobiota.77.83205.suppl1

Supplementary material 2

Table S2

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Data type: phylogenetic

Explanation note: Primers and PCR conditions used in this study. The amplification of 16S, COI and CYTB conditions.

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Link: https://doi.org/10.3897/neobiota.77.83205.suppl2

Supplementary material 3

Table S3

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Data type: word

Explanation note: The available nursery trade volumes from 2011 to 2020 were imported from all the candidate regions to mainland China from different databases.

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